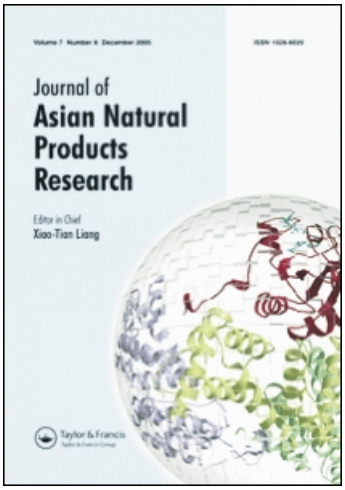

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ORIGINAL ARTICLE

Antioxidant phenolics from *Broussonetia papyrifera* fruits

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Fractionation of the EtOH extract from the fruits of *Broussonetia papyrifera* led to the isolation of 15 phenolic compounds (**1**–**15**). Their structures were identified using spectroscopic methods. Among these compounds, **1** and **2** are new and **3**–**15** were isolated from this plant for the first time. Antioxidant activities of compounds **2**–**15** against H_2O_2-induced injury in SY5Y cells and 1,1-diphenyl-2-picrylhydrazyl radical scavenging activities were evaluated.

Keywords: *Broussonetia papyrifera*; phenolics; antioxidation

1. Introduction

The fruits of *Broussonetia papyrifera* (Moraceae), known as 'Chu-Shi-Zi', have been used as an important tonic for the treatment of impotency in China [1]. The roots, barks, and leaves of *B. papyrifera* are used in medical applications in traditional Chinese medicine [1], and their chemical constituents and various biological activities have already been extensively studied [2]. Previous reports indicated that the fruit extract possesses potent antioxidant effects related to anti-aging [3], and could enhance learning and memory ability for Alzheimer's disease [4]. Our bioassay revealed that the EtOH extract of the fruits could protect neuronal cells from H_2O_2-induced injury. However, only the nutritional composition in the fruit was analyzed [5]. So far, only a limited number of substances have been isolated [6], and the specific phytochemicals responsible for its antioxidant activity have not been thoroughly identified. It is therefore imperative to investigate the antioxidant compounds which could be used for the treatment of certain diseases mediated by reactive oxygen species (ROS). Our chemical work led to the isolation of 15 phenolic compounds (Figure 1), in which **1** and **2** are new and others were isolated from this plant for the first time. The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity (RSA) and neuroprotective effects against H_2O_2-induced SY5Y cell injury were evaluated. In this paper, we describe the isolation, structural elucidation, and antioxidant activities of these isolates.

2. Results and discussion

Compound **1** is a new compound, which exhibited a quasi-molecular ion peak

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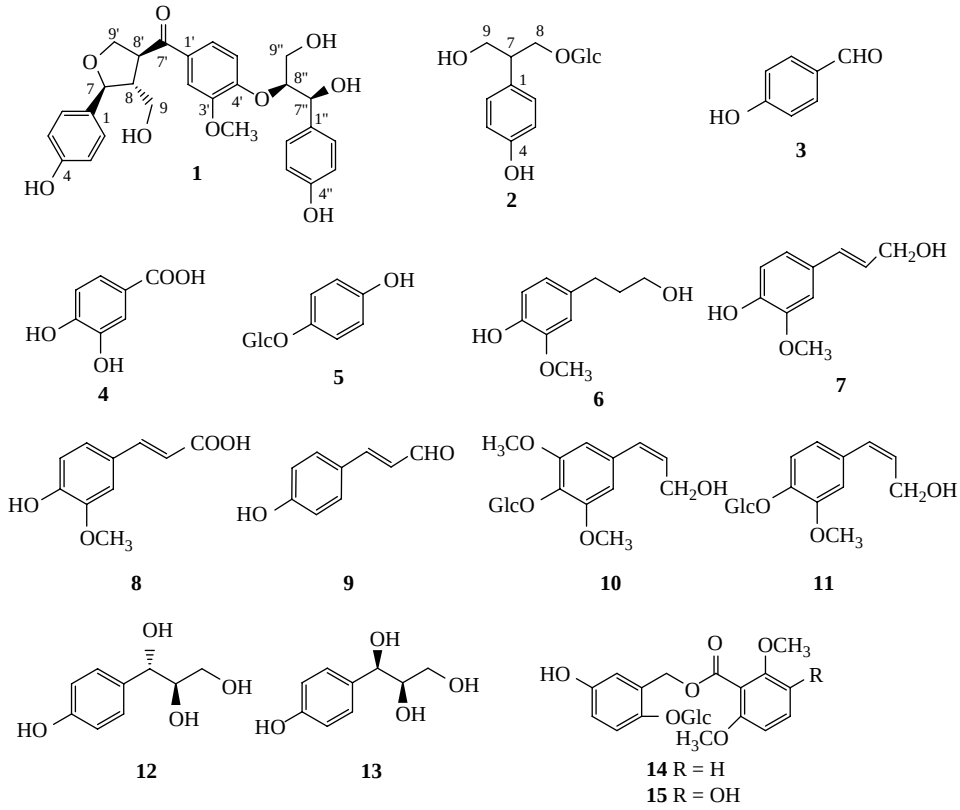

Figure 1. Structures of compounds **1**–**15**.

$[M + Na]^+$ at *m/z* 533.1783 in positive HR-ESI-MS, corresponding to the molecular formula of $C_{28}H_{30}O_9$. The ^1H NMR spectrum of **1** exhibited two typical AA′BB′ systems and an ABX system in the olefinic region (Table 1). The ^{13}C NMR spectrum of **1** showed a total of 28 carbon signals corresponding to three benzene rings, three oxymethylenes, three oxymethines, two upfield methines, a carbonyl, and a methoxy group. This evidence suggested **1** to be a sesquilignan [7], which was further confirmed by close interpretation of 2D NMR data. The $^1H-^1H$ COSY spectrum gave the spin systems of H-7 (δ 4.62)/H-8 (δ 2.67)/H-9 (δ 3.62, 3.54), H-8/H-8′ (δ 4.24)/H-9′ (δ 4.15), H-7″ (δ 4.81)/H-8″ (δ 4.57)/H-9″ (δ 3.75, 3.52). The HMBC correlations (Figure 2) of H-7/C-2, H-6′, H-9′, H-8/C-7′, and H-8″/C-1″, in combination of $^1H-^1H$ COSY and ^1H NMR spectral behaviors, revealed the presence of three phenylpropanoid units in the molecule. Additional HMBC interactions of H-7/C-9′, H-8/C-7′, H-8′/C-9, and H-8″/C-4′ allowed the linkage of the three phenylpropanoids via C-8—C-8′ and C-4′—*O*—C-8″. The OMe group was positioned at C-3′ by the aid of *O*-methyl response to C-3′ in the HMBC spectrum. The stereochemistry of **1** was achieved by NOESY correlation as well as coupling constant of vicinal protons. In the NOESY spectrum, H-7 correlating with H-8′ indicated that they are cofacial. The $J_{H\text{-}7,H\text{-}8}$ value of 8.8 Hz suggested a *trans* relationship of H-7 and H-8. The $J_{H\text{-}7'',H\text{-}8''}$ value was 6.1 Hz, indicating an *erythro*-configuration [8]. As a result, the structure of **1** was assigned

Table 1. ^{1}H and ^{13}C NMR spectral data for compound **1** (^{1}H, 400 MHz; ^{13}C, 100 MHz; in CD_3OD).

No.	δ_C	δ_H	No.	δ_C	δ_H
1	132.7		7′	200.2	
2	129.3	7.24 (d, $J = 8.2$ Hz)	8′	50.3	4.24 (m)
3	116.1	6.76 (d, $J = 8.2$ Hz)	9′	71.7	4.15 (m)
4	158.5				
5	116.1	6.76 (d, $J = 8.2$ Hz)	1″	133.2	
6	129.3	7.24 (d, $J = 8.2$ Hz)	2″	129.3	7.24 (d, $J = 8.5$ Hz)
7	85.1	4.62 (d, $J = 8.8$ Hz)	3″	116.0	6.67 (d, $J = 8.5$ Hz)
8	54.4	2.67 (m)	4″	158.0	
9	61.0	a: 3.62 (m)	5″	116.0	6.67 (d, $J = 8.5$ Hz)
		b: 3.54 (m)	6″	129.3	7.24 (d, $J = 8.5$ Hz)
1′	131.5		7″	73.2	4.81 (d, $J = 6.1$ Hz)
2′	112.8	7.54 (br s)	8″	85.3	4.57 (m)
3′	151.4		9″	62.0	a: 3.75 (m)
4′	154.3				b: 3.52 (m)
5′	115.7	7.03 (d, $J = 7.0$ Hz)	OCH_3	56.5	3.85 (s)
6′	124.3	7.57 (d, $J = 7.0$ Hz)			

as (7*R**,8*S**,8′*R**)-7″,8″-*erythro*-3′-methoxy-7′-oxo-4,4″,7″,9,9″-pentahydroxy-4′,8″:7,9′-bis-epoxy-8,8′-sesquineolignan.

Compound **2** had the molecular formula $C_{15}H_{22}O_8$ derived from its positive HR-ESI-MS at *m/z* 353.1213 $[M + Na]^+$. The ^{1}H NMR spectrum of **2** exhibited a typical AA′BB′ system in the aromatic region. The ^{13}C NMR and DEPT spectra showed signals for an aromatic ring, a sugar moiety corresponding to a glucopyranose, two oxymethylenes (δ_C 72.5, 64.9), and one methine (δ_C 49.0). The multiplicities of H-7, H-8, and H-9 indicated the presence of a 7-deoxyglycerol residue, which connected to the benzene ring via C-1—C-7 by the observed HMBC connectivity of H-2/C-7. Acid hydrolysis of **2** yielded D-glucose determined by comparison with an authentic sample and its positive optical sign in water. Furthermore, the HMBC correlation of H-1′/C-8 assigned the position of the glucosyl moiety at C-8 (Figure 2). The configuration of C-1′ was determined to be β by a $J_{H\text{-}1',H\text{-}2'}$ value of 7.9 Hz. Consequently, the structure of **2** was elucidated as 2-(4-hydroxyphenyl)propane-1,3-diol-1-*O*-β-D-glucopyranoside.

The known compounds were identified as 4-hydroxybenzaldehyde (**3**), 3,4-dihydroxybenzoic acid (**4**), arbutine (**5**) [9], dihydroconiferyl alcohol (**6**) [10], coniferyl alcohol (**7**) [11], ferulic acid (**8**) [12], *p*-coumaraldehyde (**9**), *cis*-syringin (**10**) [13], *cis*-coniferin (**11**) [14], *erythro*-1-(4-hydroxyphenyl)glycerol (**12**) [15],

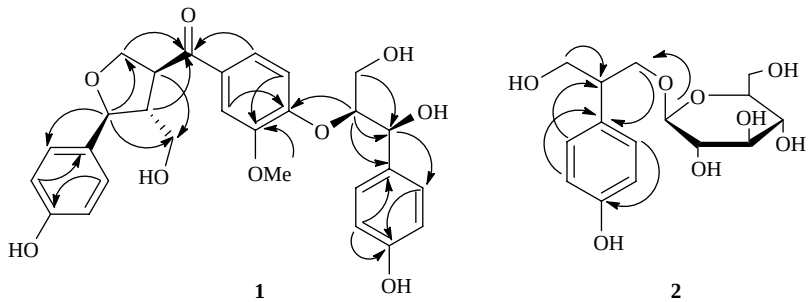

Figure 2. Important HMBC correlations for compounds **1** and **2**.

threo-1-(4-hydroxyphenyl)glycerol (**13**) [16], curculigoside I (**14**) [17], and curculigoside C (**15**) [18] by comparison of their spectroscopic data with literature values or direct interpretation of spectral data. All these compounds were isolated from this plant for the first time.

3. Experimental

3.1 General experimental procedures

Optical rotations were recorded on a Horiba SEPA-300 polarimeter. The UV spectra were measured on a Shimadzu UV-2401PC spectrophotometer. The IR spectrum was obtained on a Tensor 27 spectrometer, with KBr pellet. The NMR spectra were recorded on a Bruker AV-400 or DRX-500 spectrometer. FAB-MS were recorded on a VG Auto Spec-3000 spectrometer, and HR-ESI-MS were determined on an API QSTAR Pulsar 1 spectrometer. Column chromatography (CC) was performed on silica gel (200–300 mesh; Qingdao Marine Chemical Inc., Qingdao, China), RP-18 (40–60 μm; Daiso Co., Osaka, Japan), and Sephadex LH-20 (Amersham Pharmacia, Uppsala, Sweden). Semi-preparative HPLC was carried out on an Agilent 1100 liquid chromatography with a Zorbax SB-C_{18} column (9.4 × 250 mm, i.d.). Vitamin C, DPPH, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St Louis, MO, USA). RPMI-1640 was purchased from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum was purchased from HyClone (Logan, UT, USA). H_2O_2 was purchased from Beijing Chemical Reagents (Beijing, China). SY5Y cell line (human neuroblastoma) was obtained from the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences and Peking Union College (Beijing, China).

3.2 Plant material

The fruits of *B. papyrifera* were purchased from Yunnan Corporation of Materia Medica (YCMM), Yunnan Province, China, and identified by Mr H.Y. Sun at YCMM. A voucher specimen (CHYX0043) has been deposited at the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences.

3.3 Extraction and isolation

The dried and cracked fruits of *B. papyrifera* (30 kg) were extracted with 95% EtOH under reflux three times. The extracts were combined and evaporated to a small volume, followed by successive partition with petroleum ether, EtOAc, and BuOH. The EtOAc-soluble extract (100 g) was separated by silica gel CC with a gradient $CHCl_3$–MeOH (1:0–0:1) to afford Fr. A–E. Fr. B (12 g) was gel filtrated on Sephadex LH-20 ($CHCl_3$–MeOH 6:4) to give Fr. B_1–B_4. Fr. B_2 (3 g) was repeatedly subjected to C_{18} (MeOH–H_2O 3:7–9:1) and silica gel CC (petroleum ether–EtOAc 20:1–1:1) to afford compounds **6** (7 mg), **7** (22 mg), and **9** (2 mg). Fr. C (11 g) was fractionated on Sephadex LH-20 ($CHCl_3$–MeOH 6:4) to yield Fr. C_1–C_4. Fr. C_2 (2 g) was purified by repeated C_{18} (MeOH–H_2O 3:7–9:1) and silica gel CC (petroleum ether–EtOAc 15:1–1:1) to yield compounds **3** (50 mg) and **8** (4 mg). Fr. D (10 g) was divided into fractions D_1–D_4 by Sephadex LH-20 (MeOH). Fr. D_2 (1 g) was further purified by a combination of C_{18} (MeOH–H_2O 1:9–7:3) and silica gel CC ($CHCl_3$–MeOH 30:1–20:1) to produce **4** (60 mg). Likewise, compounds **12** (6 mg) and **13** (8 mg) were isolated from D_3 (1.2 g). The BuOH extract (50 g) was divided into Fr. F–H by silica gel CC with a gradient $CHCl_3$–MeOH (9:1–3:1). Fr. G (9 g) was passed through Sephadex LH-20 (MeOH–H_2O 8:2) to obtain Fr. G_1–G_4. Fr. G_2 (2 g), G_3 (2 g), and G_4 (3 g) were, respectively, separated by C_{18} (MeOH–H_2O 1:9–1:1) followed by semi-preparative HPLC

(MeOH–H_2O 3:7) to obtain compounds **1** (7 mg), **10** (6 mg), and **11** (4 mg) from G_2, **2** (15 mg) and **14** (20 mg) from G_3, and **5** (12 mg) and **15** (22 mg) from G_4.

3.3.1 (7R,8S*,8′R*)-7″,8″-Erythro-3′-methoxy-7′-oxo-4,4″,7″,9,9″-pentahydroxy-4′,8″:7,9′-bis-epoxy -8,8′-sesquineolignan (**1**)*

Colorless solid, $[\alpha]_D^{21.6} = -2.01$ ($c = 0.10$, MeOH). UV (MeOH) λ_{max} (log ε): 306 (3.74), 277 (3.92), 226 (4.25), 201 (4.42) nm. ^{1}H and ^{13}C NMR (CD_3OD): see Table 1. FAB-MS: *m/z* 511 $[M + H]^+$. HR-ESI-MS: *m/z* 533.1783 $[M + Na]^+$ (calcd for $C_{28}H_{30}O_9Na$, 533.1787).

*3.3.2 2-(4-Hydroxyphenyl)propane-1,3-diol-1-O-β-D-glucopyranoside (**2**)*

Colorless solid, $[\alpha]_D^{27.6} = +18.0$ ($c = 0.10$, MeOH). UV (MeOH) λ_{max} (log ε): 277 (3.19), 223 (3.86), 203 (3.77) nm. IR (KBr): 3385, 2944, 2882, 2504, 1413, 1518, 1451, 1080, 1028, 966, 832 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.09 (d, $J = 8.5$ Hz, H-2, 6), 6.71 (d, $J = 8.5$ Hz, H-3, 5), 2.98 (m, H-7), 4.09 (dd, $J = 11.0$, 5.6 Hz, Ha-8), 3.79 (m, Hb-8), 3.85 (m, Ha-9), 3.75 (m, Hb-9), 4.29 (d, $J = 7.9$ Hz, H-1′), 3.18 (m, H-2′), 3.27 (m, H-3′, 4′), 3.35 (m, H-5′), 3.85 (m, Ha-6′), 3.65 (dd, $J = 14.8$, 7.6 Hz, Hb-6′). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 132.7 (C-1), 130.3 (C-2, 6), 116.1 (C-3, 5), 157.1 (C-4), 49.0 (C-7), 72.5 (C-8), 64.9 (C-9), 104.7 (C-1′), 75.0 (C-2′), 77.9 (C-3′), 71.5 (C-4′), 78.0 (C-5′), 65.0 (C-6′). FAB-MS: *m/z* 329 $[M - H]^-$. HR-ESI-MS: *m/z* 353.1213 $[M + Na]^+$ (calcd for $C_{15}H_{22}O_8Na$, 353.1212).

3.4 Acid hydrolysis of compound 2

Compound **2** (6 mg) was dissolved in 2 M HCl (5 ml) and heated in a water bath at 70°C for 6 h. After cooling, the reaction mixture was neutralized and extracted with $CHCl_3$. TLC comparison (silica gel, $CHCl_3$–MeOH 6:4) with authentic samples revealed the presence of glucose in the water layer. The D-form of glucose was determined by its positive optical rotation in water.

3.5 DPPH radical scavenging assay

The DPPH assay was carried out according to a previously described method [19]. Briefly, 10 μl of different concentrations of the tested compounds (final concentrations ranging from 0.16 to 100 μM) was added to 190 μl of DPPH solution (0.1 mM in EtOH), followed by 30 min of reaction at room temperature. The absorbance of the solution was read at 517 nm with a spectrophotometer (M5; Molecular Device Corporation, Sunnyvale, CA, USA). The percentage of RSA (RSA%) was calculated as follows: $RSA\% = [(A_c - A_t)/A_c] \times 100\%$, where A_c is the average absorbance of the control and A_t is the absorbance of the tested compounds or positive drug. In this assay, vitamin C was used as a positive control; the tests were performed in triplicate.

3.6 Antioxidant assay against H_2O_2-induced injury in SY5Y cells

SH-SY5Y cells were grown in RPMI-1640 supplemented with 5% fetal bovine serum, 10% horse serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 20 mM L-glutamine. SY5Y cell suspensions, which were adjusted to 1×10^6/ml, were seeded into a 96-well culture plate at 100 μl/well and incubated at 37°C, 5% CO_2 for 24 h, followed by incubation with H_2O_2 (final concentration of 250 μM) and different concentrations of compounds (final concentrations of 0.16–100 μM) for another 24 h. After the treatment, cell viability was measured by the MTT method [20]. In brief, cells in the 96-well plate were rinsed with serum-free RPMI-1640. MTT (0.5 mg/ml) was added to each

Table 2. Radical scavenging capacity of compounds **2**–**15** against DPPH.

	RSA%					
Group	0.16 μM	0.8 μM	4 μM	20 μM	100 μM	IC_{50} (μM)
Control	–					–
2	2.12 ± 0.33	2.67 ± 0.30	2.13 ± 0.31	1.82 ± 0.54	1.12 ± 0.26	>500
3	1.15 ± 3.75	0.22 ± 3.81	3.75 ± 3.62	−2.56 ± 2.98	6.24 ± 1.84	>500
4	6.43 ± 2.82	1.50 ± 1.54	21.48 ± 3.43	64.88 ± 2.11	82.36 ± 1.02	39.52
5	2.15 ± 2.14	1.26 ± 1.71	3.24 ± 1.21	1.66 ± 1.17	11.17 ± 1.07	>500
6	4.36 ± 1.83	4.77 ± 1.48	15.00 ± 2.16	36.87 ± 2.27	71.34 ± 1.17	58.89
7	5.10 ± 1.26	4.80 ± 1.28	14.87 ± 5	23.23 ± 2.13	53.19 ± 1.59	87.07
8	4.77 ± 0.91	4.49 ± 0.03	9.88 ± 1.90	34.89 ± 2.10	65.41 ± 1.35	65.32
9	−3.31 ± 2.11	−3.43 ± 1.78	−3.65 ± 1.42	−3.21 ± 0.31	3.57 ± 2.31	>500
10	−2.65 ± 1.11	−2.41 ± 0.31	−2.32 ± 3.01	−1.89 ± 0.97	1.07 ± 0.84	>500
11	9.88 ± 0.25	10.28 ± 2.26	12.40 ± 3.09	9.15 ± 2.01	20.63 ± 0.61	>300
12	−3.50 ± 0.81	−0.74 ± 3.77	−5.03 ± 0.06	−4.86 ± 0.93	−5.11 ± 2.10	>500
13	−4.54 ± 1.97	−3.76 ± 1.10	−5.44 ± 1.30	−5.04 ± 0.79	−4.86 ± 1.75	>500
14	0.08 ± 1.31	2.75 ± 1.35	0.24 ± 1.66	3.34 ± 0.64	16.52 ± 2.93	>300
15	2.07 ± 0.27	3.56 ± 1.98	9.58 ± 0.84	29.32 ± 1.00	69.02 ± 0.36	65.6
Vitamin C	−0.08 ± 3.73	4.02 ± 0.23	15.86 ± 1.54	50.90 ± 1.43	84.61 ± 0.86	46.20

Note: [a]Values represent mean ± SD of three replicates.

Table 3. Antioxidant effects of compounds **2**–**15** against H_2O_2-induced injury in SY5Y cells.

	Percentage inhibition				
Group	0.16 μM	0.8 μM	4 μM	20 μM	100 μM
Control	100*				
Model	23.07 ± 2.82				
2	27.12 ± 1.20	27.83 ± 2.02	29.95 ± 1.90***	27.41 ± 0.42	27.80 ± 7.20
3	20.80 ± 4.47	30.26 ± 4.45***	23.96 ± 2.57	26.91 ± 2.90	24.13 ± 4.32
4	18.57 ± 2.10	30.28 ± 2.19***	28.43 ± 0.17***	37.79 ± 2.56**	34.70 ± 0.85**
5	19.55 ± 0.66	26.18 ± 1.07	25.23 ± 2.36	24.12 ± 0.04	24.29 ± 0.69
6	27.98 ± 1.97	25.41 ± 2.91	27.50 ± 3.19	21.33 ± 3.16	18.61 ± 3.17
7	25.02 ± 2.33	30.42 ± 1.52***	27.62 ± 6.94	23.45 ± 1.33	11.22 ± 1.78**
8	22.80 ± 0.18	22.44 ± 1.86	19.30 ± 2.31	23.57 ± 3.05	25.05 ± 0.60
9	20.64 ± 4.54	21.68 ± 0.17	23.46 ± 1.31	27.98 ± 1.16***	18.79 ± 1.02
10	21.47 ± 3.91	23.52 ± 3.49	26.13 ± 2.86	32.79 ± 8.98	29.52 ± 6.18
11	24.60 ± 0.92	26.52 ± 0.47	25.20 ± 0.15	24.56 ± 1.04	25.62 ± 3.82
12	25.99 ± 1.47	25.35 ± 1.26	26.61 ± 1.96	26.19 ± 0.60	23.42 ± 3.23
13	26.16 ± 1.33	23.64 ± 1.62	24.58 ± 0.42	16.63 ± 0.55***	20.84 ± 2.11
14	25.90 ± 1.65	24.62 ± 3.61***	26.84 ± 1.68	34.41 ± 5.80***	26.27 ± 1.86
15	35.83 ± 1.08	37.27 ± 3.69	36.33 ± 1.86	39.62 ± 0.57**	34.46 ± 4.42***
Vitamin C	27.07 ± 4.68	24.88 ± 3.48	30.88 ± 5.47***	29.72 ± 6.43	29.84 ± 3.52**

Note: *$P < 0.001$, **$P < 0.01$, ***$P < 0.05$ vs. model.
[a]Values represent mean ± SD of six replicates.

well and incubated for 4 h at 37°C. After the medium with MTT was removed, 200 μl of DMSO was added to each well. Optical density was measured at 570 nm on a microplate reader (Molecular Devices Corporation).

Antioxidant activities of compounds **2**–**15** were examined using DPPH radical scavenging assay and H_2O_2-induced injury in SY5Y cell assay. As shown in Table 2, several isolates showed radical scavenging capacity against DPPH. Among these isolates, the IC_{50} values of compounds **4**, **6**, **8**, and **15** were 39.5, 58.9, 65.3, and 65.6 μM, respectively, and they were comparable to that of vitamin C, a well-known antioxidant. As indicated by the IC_{50} values, the DPPH radical scavenging abilities of compounds **4**, **6**–**8**, and **15** with two vicinal oxygen-bearing groups at the benzene ring are much stronger than those of **2**, **3**, **5**, **9**, **12**–**14** lacking this structure motif in the molecules. The presence of OH or OMe at C-3 may activate the site of the O—H bond at the C-4 position, which led to an increase in H-donation. In the neuroprotective assay (Table 3), the results showed that compounds **2**–**4**, **7**, **9**, **13**–**15** could salvage SY5Y cell death induced by H_2O_2, a typical ROS. Particularly, compounds **4** and **15** displayed better neuroprotective effects at a concentration of 20 μM.

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Note

1. These authors contributed equally to this paper.

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